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Synthesis and multimodal activity of 3a,6-epoxyisoindole-2(3*H*)-(carbox/thio/seleno)amides in models of glycation, oxidative stress, and inflammation: Toward the development of agents targeting the triggering mechanisms of fibrogenesis

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The aim. Within a series of 3a,6-epoxyisoindole-2*(3H)*-(carbox/thio/seleno)amides, we sought to identify a multimodal scaffold suitable for the further development of agents to prevent and treat fibrotic diseases by assessing of compound's ability to mitigate glycation and oxidative stress, key triggers of fibrogenesis; to select a non-cytotoxic lead with a balanced combination of these two activities, and to preliminarily evaluate its anti-inflammatory potential.

Materials and methods. Target 3a,6-epoxyisoindole-2(*3H*)-(carbox/thio/seleno)amides were synthesised using the IMDAF approach. Antiglycation activity was evaluated in a bovine serum albumin-glucose model by registering advanced glycation end-product (AGE) fluorescence. Antioxidant properties were determined using the ABTS assay. Cytotoxicity and anti-inflammatory effects were studied in peritoneal macrophages from adult wild-type white mice (n = 4; body mass 30–35 g). Cytotoxicity was assessed by the MTT assay and lactate dehydrogenase (LDH) release, while anti-inflammatory effects were evaluated in a model of LPS-induced nitric oxide (NO) production.

Results. The study delineates promising directions for modifying the epoxyisoindole scaffold for drug discovery and proposes a screening framework for agents targeting pathologies dependent on non-enzymatic damaging mechanisms (glycation, oxidation), including fibrotic diseases. Active molecules were identified among derivatives of hydrogenated 3a,6-epoxyisoindole. Compound **2.10** — 7a-chloro-*N*-(4-chlorophenyl)-1,6,7,7a-tetrahydro-3a,6-epoxyisoindole-2(*3H*)-carbothioamide — exhibited an optimal balance of antiglycation (at 100 μM, inhibition of glycation 40.1 ± 1.7%) and antioxidant activity (at 111 μM, reduction in ABTS•+ colour intensity 57.1 ± 1.1%) with low cytotoxicity (apparent from ≥ 250 μM). By contrast, compounds **2.16**–**2.19** (bearing an aroyl fragment) showed exceptionally high antioxidant activity (95.0–96.5% reduction in ABTS•+ colour intensity) without concordant antiglycation effects (inhibition not exceeding 15%). In the model used, anti-inflammatory activity of **2.10** was not detected.

Conclusion. Compound **2.10** is a promising starting point for further structural optimisation toward agents acting on early pathogenetic events driven by non-enzymatic damaging triggers, including the prevention and treatment of fibrotic remodelling.

Keywords: epoxyisoindole; antiglycation; antioxidant activity; inflammation; fibrosis; macrophages

Abbreviations: ABTS — 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); DMEM — Dulbecco's Modified Eagle Medium; IMDAF — IntraMolecular Diels-Alder Furan reaction; BSA — bovine serum albumin; DMSO — dimethyl sulfoxide; AGE — advanced glycation end products; LDH — lactate dehydrogenase; LPS — lipopolysaccharide; NAD+ — nicotinamide adenine dinucleotide (oxidized form of the molecule); NADH — nicotinamide adenine dinucleotide (reduced form of the molecule); PMs — peritoneal macrophages; TLC — thin-layer chromatography; NMR — nuclear magnetic resonance.

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Синтез и мультимодальная активность 3а,6-эпоксиизоиндоло-2(3*H*)-(карбокс/тио/селен)амидов в моделях реакции гликирования, окислительного стресса и воспаления, ориентация на разработку средств, воздействующих на триггерные механизмы фиброзирования

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Цель. В ряду 3а,6-эпоксиизоиндол-2(3*H*)-(карбокс/тио/селен)амидов выявить мультимодальный скаффолд, пригодный в качестве основы для дальнейшей разработки средств профилактики и терапии фиброзных заболеваний; оценить антигликирующую и антиоксидантную активность ряда соединений, отобрать нецитотоксичное соединение-лидер со сбалансированным сочетанием двух активностей и предварительно проверить его противовоспалительное действие.

Материалы и методы. Целевые 3а,6-эпоксиизоиндол-2(3*H*)-(карбокс/тио/селен)амиды синтезированы с использованием IMDAF-реакции. Антигликирующую активность оценивали в модели гликирования альбумина глюкозой, регистрируя флуоресценцию конечных продуктов гликирования (КПГ). Антиоксидантные свойства определяли с применением ABTS. Цитотоксическое и противовоспалительное действие изучали на перитонеальных макрофагах белых половозрелых мышей дикого типа (*n*=4, масса 30–35 г). Цитотоксичность оценивали с использованием МТТ-теста и по высвобождению лактатдегидрогеназы (ЛДГ), противовоспалительный эффект — в модели ЛПС-индуцированной продукции оксида азота (NO).

Результаты. В результате работы показаны перспективные направления модификации эпоксиизоиндольного скаффолда для разработки новых лекарственных препаратов; предложена система поиска средств для профилактики и лечения патологий, зависимых от пусковых механизмов повреждения гликированием и окислительным стрессом, в том числе фиброзных болезней. Идентифицированы активные молекулы (производные гидрированного 3а,6-эпокиизоиндола). Соединение **2.10**, а именно 7а-хлор-*N*-(4-хлорфенил)-1,6,7,7а-тетрагидро-3а,6-эпокиизоиндол-2(3*H*)-карботиоамид, продемонстрировало оптимальное сочетание антигликирующей (в концентрации 100 мкМ ингибирование реакции гликирования на 40,1±1,7%) и антиоксидантной активности (в концентрации 111 мкМ снижение интенсивности окраски ABTS•+ на 57,1±1,1%) при низкой цитотоксичности (проявляется в концентрациях ≥250 мкМ), тогда как **2.16–2.19** (содержат структурный N-ароильный фрагмент) отличались исключительно высокой антиоксидантной активностью (снижение интенсивности окраски ABTS•+ на 95,0–96,5%) без согласования таковой с антигликирующим действием (для лучшего соединения не достигает 15% ингибирования реакции гликирования). Противовоспалительная активность **2.10** в использованной модели выявлена не была.

Заключение. Соединение **2.10** — перспективная основа для дальнейшей оптимизации структуры в направлении создания средств, ориентированных на ранние звенья патогенеза заболеваний, зависимых от механизмов повреждения гликированием и окислительным стрессом, в том числе для профилактики и лечения фиброзного ремоделирования.

Ключевые слова: эпоксиизоиндол; антигликирование; антиоксидантная активность; воспаление; фиброз; макрофаги

Список сокращений: ABTS — 2,2’-азино-бис(3-этилбензотиазолин-6-сульфоновая кислота); DMEM — модифицированная Дульбекко среда Игла; IMDAF — внутримолекулярная реакция Дильса-Альдера с участием фурана; БСА — бычий сывороточный альбумин; ДМСО — диметилсульфоксид; КПГ — конечные продукты гликирования; ЛДГ — лактатдегидрогеназа; ЛПС — липополисахарид; НАД+ — никотинамидадениндинуклеотид (окисленная форма молекулы); НАДН — никотинамидадениндинуклеотид (восстановленная форма молекулы); ПМ — перитонеальные макрофаги; ТСХ — тонкослойная хроматография; ЯМР — ядерный магнитный резонанс.

INTRODUCTION

At the heart of many difficult-to-treat, debilitating diseases lie the processes of pathological fibrotic remodeling of tissues [1]. One of the leading roles in the development of fibrosis is assigned to the persistent transition of fibroblasts into myofibroblasts [2], inflammation [3], changes in the expression of signaling molecules (for example, FGF23 in the heart, interleukins IL-6 and IL-11) [4–6], as well as an imbalance in the degradation and synthesis of extracellular matrix components [7]. At the same time, the above conditions, despite their high pathogenetic significance, are reactive, that is, capable of developing in response to the action of a triggering damaging mechanism [8–10]. Such triggering mechanisms include damage to molecules by factors such as glycation and oxidative stress [9, 10].

Focusing on fibrosis as a pathology that is difficult to treat, as well as the secondary nature of many significant pathogenetic mechanisms of fibrotic remodeling, it can be concluded that targeting the earliest triggering links in pathogenesis is a promising strategy in the prevention and treatment of the disease [11]. Moreover, acting on several mechanisms at once may have even greater success [12]. In general terms, an approach to the search for new antifibrotic compounds, based on assessing the effect of such compounds on both early, triggering, and later, secondary mechanisms of fibrotic remodeling, can serve as an effective strategy for finding new antifibrotic drugs.

THE AIM. To analyze and identify in the series of 3a,6-epoxyisoindole-2(3H)-(carbox/thio/seleno)amides a multimodal scaffold suitable as a basis for further development of means for the prevention and therapy of fibrotic diseases; to evaluate the antiglycation and antioxidant activity of a number of compounds, to select a non-cytotoxic lead compound with a balanced combination of two activities and to preliminarily test its anti-inflammatory potential.

MATERIALS AND METHODS

Synthesis of compounds

Series of compounds **1.1–1.12** and **2.1–2.19** for biological tests were synthesized using thermal intramolecular Diels-Alder reaction in the furan derivatives (IMDAF reaction), which is often used both for the directed production of alkaloids [13–16] and for studying the fundamental laws of organic reactions [17–19].

At the first stage, the corresponding *N*-allylfurfurylamines were obtained by reductive amination using one of two methods (Fig. 1), depending on the loading, as described in [20–23].

Method A (mass of the initial furfural is less than 5 g). An equimolar amount of allylamine (all commercially available reagents were provided by Thermo Scientific Chemicals) was added to a solution of furfural in dichloromethane, after which the reaction mixtures were stirred at room temperature in the presence of MS 3Å molecular sieves for 24 hours (TLC control). After removing the solvent, the resulting oils were dissolved without further purification in methanol (with the addition of tetrahydrofuran in case of incomplete dissolution), and a two-fold molar excess of sodium borohydride was added at room temperature with constant stirring. After incubation for 24 hours and standard workup, the secondary amines were isolated as pale yellow oils by column chromatography (SiO_2, eluent: hexane-ethyl acetate) with a yield of 40–50%.

Method B (mass of the initial furfural is more than 5 g). An equimolar amount of allylamine was added to a solution of furfural in benzene, after which the mixture was boiled with a Dean-Stark trap until the theoretical amount of water was separated. After evaporation of benzene, the resulting oils were dissolved without further purification in ethanol and reacted with sodium borohydride (2 eq) at boiling. After standard workup, the resulting oils were purified by fractionation under reduced pressure. The target amines were obtained as colorless or light yellow oils with yields of 45–50%.

The synthesis of epoxyisoindolecarbo(thio, seleno)amides **1.1–1.12** and **2.1–2.19** was carried out in benzene or toluene, as described in [24, 25] (Fig. 2 and Fig. 3). The corresponding iso(thio,seleno)cyanate (R^3NCO or R^3NCX) was added to a solution of the furfurylallylamines obtained above, and the reaction mixture was boiled for 6–8 hours (TLC control, Sorbfil, hexane : ethyl acetate 80 / 20). After cooling, the resulting crystals were filtered off, washed with ether, to obtain the target products as colorless crystals with yields of 28–93%.

The significant difference in the yields of the target 3a,6-epoxyisoindoles is due to the different steric load of the diene and dienophile in the transition state of the intramolecular [4+2] cycloaddition, as well as the different basicity of the secondary nitrogen atom in the initial *N*-allylfurfurylamines (which affects the rate of nucleophilic addition of isocyanate). It has been shown that bulky aliphatic substituents at the nitrogen atom of *N*-allylfurufuryl(thio,seleno) ureas reduce the yields of Diels-Alder adducts. The

ОРИГИНАЛЬНАЯ СТАТЬЯ
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moderate yield of selenoureas **2.12–2.15** (Fig. 3) is explained by the fact that prolonged heating of the reaction mixtures in benzene leads to the destruction of products with the formation of a red selenium precipitate.

The structure of the obtained products and their purity were confirmed by ^{1}H, ^{13}C NMR, mass spectrometry and elemental analysis. The obtained spectral data correlate with those described previously in [24, 25].

Antiglycation activity *in vitro*

The glycation reaction was carried out in a 0.05M phosphate buffer solution, pH 7.4. Composition of the reaction medium: glucose 0.36 M (Agat-Med, Russia), BSA (fraction V) 1 mg/mL (Sigma, USA). The test compounds were dissolved in 99% DMSO (Kemerovo FF, Russia) (final concentration of the solvent in the reaction medium ~3%). The activity of the compounds was studied at a concentration of 100 µM. Control samples contained an equivalent volume of solvent. The samples were incubated for 24 hours at 60°C. Data were recorded by a spectrofluorimetric method, determining advanced glycation end products (AGEs) by their specific fluorescence at excitation/emission wavelengths of 440 / 520 nm (Infinite M200 PRO microplate reader, Tecan, Austria).

In order to exclude false-positive results for compounds that suppress AGE fluorescence due to interference, logarithmic normalization of the obtained data was performed using formula 1:

$$Flu(lg) = 10^{lg(exp) - lg(Blank)} - 1,$$

where Flu(lg) is the normalized fluorescence intensity of AGEs; lg(exp) and lg(blank) are the decimal logarithms of the actual fluorescence levels of glycated and corresponding non-glycated samples (both containing the test compound and control).

The activity of other compounds (both non-fluorescent and fluorescent at the wavelengths used) was expressed by formula 2:

$$Flu(lin) = Exp - Blank,$$

where Flu(lin) is the fluorescence intensity of AGEs; Exp and Blank are the actual fluorescence levels of glycated and corresponding non-glycated samples (both containing the test compound and control).

The determination of activity, expressed as % suppression of AGE fluorescence, was performed using formula 3:

$$\% = 100 - \left(Flu(exp) * \frac{100}{Flu(Contr)} \right),$$

where Flu(Exp) and Flu(Contr) are the fluorescence intensity of AGEs in experimental and control samples, respectively (log-normalized or non-log-normalized).

In vitro antioxidant properties

ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); «Sigma», Canada) is a chromogenic substrate used for the quantification of antioxidant activity. In the process of oxidation of this substrate, a stable ABTS•+ radical is formed, characterized by an intense blue-green color. Antioxidants are able to reduce this radical, which leads to a decrease in color intensity, thereby allowing the antioxidant capacity of the compounds under study to be assessed. The radical is generated by adding a hydrogen peroxide-hemoglobin system to the reaction medium. Hemoglobin («Serva», Germany) was dissolved in 0.1M phosphate buffer solution (pH=6.8) to a final concentration of 1 mg/mL. ABTS was prepared at a concentration of 0.4 mg/ml by diluting 2 mg in 1 mL of PBS. Hydrogen peroxide was prepared by mixing 197 µL of 3% H_2O_2 («Ivanovo Pharmaceutical Factory», Russia) with 9833 µL of distilled water, reaching a concentration of 0.05%. Spectrophotometric determination of the optical density of the medium was carried out at a wavelength of 734 nm using a Tecan Infinite M200 PRO multifunctional microplate reader (Tecan, Austria).

Ethics approval

The manipulations were carried out in accordance with the requirements of ARRIVE 2.0. Ethical approval was obtained on 10/23/2024 from the Local Ethics Committee of the Volgograd State Medical University (registration number IRB 00005839, IORG 0004900 [OHRP]).

Assessment of the cytotoxicity of the lead compound, as well as its anti-inflammatory properties upon induction of inflammation by bacterial lipopolysaccharide

Primary mouse peritoneal macrophages (PMs) were used as target cells. PMs were isolated from peritoneal exudate of wild-type white sexually mature mice (*n* = 4, weight 30–35 g). The animals were obtained from the bio-nursery of LLC "SMK STEZAR" (Vladimir) and underwent a 2-week quarantine in the vivarium of the Volgograd State Medical University. The maintenance of animals and the conduct of experiments complied with the "Principles of Good Laboratory Practice" (GOST R-53434-2009) and the recommendations of the "Guidelines for

Preclinical Studies of Medicines". To stimulate the accumulation and activation of PMs, mice were injected intraperitoneally with 1 mL of 3% peptone solution. After 3 days, the animals were humanely euthanized by cervical dislocation. Peritoneal cells were collected by aseptic washing of the abdominal cavity with 5 ml of sterile Hanks' solution (PanEco, Russia) (without calcium and magnesium ions) at a temperature of 4–6°C. The resulting lavage was centrifuged at 250 g for 10 min (SIGMA 2-16KL centrifuge, Germany), the supernatant was removed, and the pellet was resuspended to obtain a cell suspension, followed by centrifugation (5 min) and resuspension. The total number of cells was counted and their viability was assessed in a Goryaev counting chamber (Russia) with staining with 0.4% trypan blue («Sigma-Aldrich», USA). The cell concentration was adjusted to 2.0×106 cells/mL in complete DMEM nutrient medium (Gibco) supplemented with 2 mM L-glutamine (Gibco), 10% heat-inactivated fetal bovine serum («BioClot», Germany) with the addition of 100 U/mL penicillin and 100 mg/mL streptomycin (Gibco). The cells were seeded into 96-well plates at 200 µL per well and incubated for 2 h at 37°C in a humidified atmosphere (95% humidity) with 5% CO2, after which the wells were washed with Hanks' solution (without calcium and magnesium ions) to remove unattached cells. After 24 h of incubation, 40 µL of supernatant was taken from each well, and 20 µL of solutions of the tested substances were added in the concentration range of 10–10000 µM, which provided a final concentration of 1–1000 µM in the well. The range of selected concentrations is acceptable and is found in the literature in various combinations of specific studied concentrations [26–28]. Incubated for 30 min in a CO_2 incubator. After 30 min, 20 µL of lipopolysaccharide (LPS) E. coli O26:B6 (in DMEM medium) («Sigma-Aldrich», USA) (C = 100 ng/well) was added and incubated for 24 hours. After co-incubation of PMs with the test and control substances, 20 µL of cell supernatant was taken for the LDH test and 50 µL of cell supernatant was taken to determine NO production. The remaining culture plate with PMs was used for further MTT testing.

Performing the MTT assay

A colorimetric MTT assay was used to assess cell viability. After 24 hours of exposure to the compounds and aspiration of 70 µL of cell suspension, the cells were treated with MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) («Sigma», China) at a concentration of 5 mg/mL in PBS in a ratio of 1:10 and incubated for another 2.5 h. At the end of the incubation, the MTT solution was removed, 150 µL of DMSO («Kemerovo Pharmaceutical Factory», Russia) was added to dissolve the formazan crystals, and the plates were shaken for 5 min. The optical density was measured at a wavelength of 565 nm using a Tecan Infinite M200 PRO multifunctional microplate reader (Tecan, Austria). The selected wavelength corresponds to the absorption maximum and provides optimal sensitivity; similar parameters have been described previously [29].

Performing the LDH assay

An increase in the level of the enzyme lactate dehydrogenase (LDH) in the culture medium indicates a violation of the integrity of the cell membrane and cell death. To measure the LDH content in supernatants, a method was used based on spectrophotometric monitoring of the decrease in NADH concentration in the presence of pyruvate. 20 µL of supernatants taken 24 hours after incubation of PMs with the test and control compounds (final concentration 1–1000 µM) were mixed with 250 µL of NADH solution (PanEco, Russia) at a concentration of 0.194 mM/l, dissolved in 54 mM phosphate buffer, pH=7.5. Then, 25 µL of pyruvate solution (PanEco, Russia) at a concentration of 6.48 mM was added to the mixture.

The change in optical density was recorded at a wavelength of 340 nm for 20 min using a Tecan Infinite M200 PRO multifunctional microplate reader (Tecan, Austria). The selected wavelength corresponds to the peak absorption of NADH, providing optimal sensitivity for its quantification [30]. This time is necessary for the enzymatic reaction of pyruvate conversion to lactate and NADH oxidation to NAD+, which underlies this test system [31]. To determine cell viability, a standard curve was used, where the viability of whole cells was taken as 100%, and those treated with Triton X-100 as 0%.

Determination of NO production by peritoneal macrophages

The expression of iNOS by cells was induced by adding a sterile solution of LPS in a volume of 20 µL, the final concentration was 100 ng/well. Dexamethasone at a concentration of 100 µM was used as a reference drug. Incubated for 24 h under standard CO_2 incubator conditions.

The accumulation of nitrite (a stable end product of NO) in supernatants was determined using a standard Griess reagent. The Griess method is based on the diazotization of the nitrite ion in an acidic medium

ОРИГИНАЛЬНАЯ СТАТЬЯ
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with sulfanilamide and the interaction of the diazo compound with *N*-(1-naphthyl)ethylenediamine to form a colored derivative. After 24 hours of incubation, 50 µL of supernatant was taken from the wells of the plate, 100 µL of reagent was added (1% sulfanilamide in 5% orthophosphoric acid and 0.1% aqueous solution of *N*-(1-naphthyl)ethylenediamine in equal proportions). The optical density was determined in a microplate reader at 550 nm using a Tecan Infinite M200 PRO multifunctional microplate reader (Tecan, Austria). The selected procedure corresponds to [32].

Statistical analysis

Statistical data processing was performed using GraphPad Prism 9.0, using the ANOVA criterion (Dunnett's post-test). The calculation of statistical parameters was carried out for linearly normalized data (normalization was carried out by subtracting the values obtained for blank samples from the results obtained for the corresponding experimental samples). Correlation analysis was performed using Pearson's correlation coefficient at a significance level of $p < 0.05$.

RESULTS

In vitro antiglycation activity

The results of the study of the antiglycation properties of the compounds are presented in Table 1.

The study identified several of the most active compounds, namely **2.5**, **2.8**, **2.10**, **2.11**, **2.12**, **2.13**, **2.14** and **2.15**. Despite a significant superiority in the level of activity over alagebrium (the latter shows activity under these experimental conditions only at a concentration of 1000 µM), the activity of the noted compounds still seems moderate, but sufficient for conducting further modifications of the structure. The negative activity value of compound **1.9** can probably be considered as a result of interference or measurement error.

In vitro antioxidant properties

The results of the study of the antioxidant properties of the compounds are presented in Table 2.

The study identified several of the most active compounds: **2.2**, **2.3**, **2.4**, **2.5**, **2.6**, **2.7**, **2.8**, **2.9**, **2.10**, **2.11**, **2.12**, **2.13**, as well as **2.16**, **2.17**, **2.18**, **2.19** (the activity of the last 4 compounds turned out to be the highest in the series).

According to a mechanistic view of the glycation reaction, the course of the reaction largely depends on oxidation processes [33], due to which antioxidant compounds may be promising antiglycators [34]. In one of our earlier works, we demonstrated a similar correlation between antiglycation activity and the ability of compounds to prevent copper-dependent ascorbate autooxidation [35]. It is noteworthy that in the case of the studied series of compounds, there is no statistically significant correlation between the two activities (Pearson's criterion, R = 0.239, p = 0.196). At the same time, the exclusion of compounds **2.16**, **2.17**, **2.18**, **2.19**, which showed the highest antioxidant properties, led to the emergence of a statistically significant correlation (Pearson's criterion, R = 0.607, p = 0.001). This correlation is graphically presented in Figure 4.

Such discrepancy in the values of the two estimated activities for these four compounds may be a consequence of interference in one of the two tests, and therefore requires further investigation. At the same time, we note that these compounds contain a structural *N*-aroyl fragment, possibly significant for the manifestation of only one activity, but not the second, even despite the connection between them. It is also possible that this antioxidant activity, due to undetermined features, is able to manifest itself only under the conditions of the ABTS test and is not significant for the course of the glycation reaction, which limits its potential usefulness. Thus, despite the presence of dramatically high antioxidant activity, comparable to the highly active compound quercetin, and considering the insufficient reliability and explainability of the results, compounds **2.16**, **2.17**, **2.18**, and **2.19** were not considered when selecting the lead. Studies of the antioxidant properties of these compounds will be continued and deepened in the future.

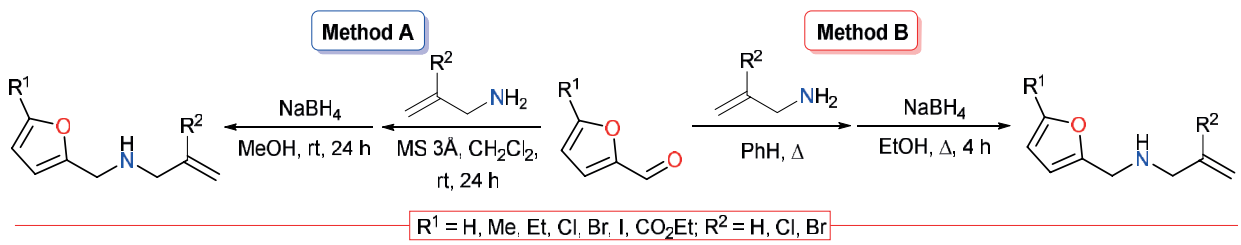

Figure 1 – Obtaining the initial *N*-allylfurfurylamines.

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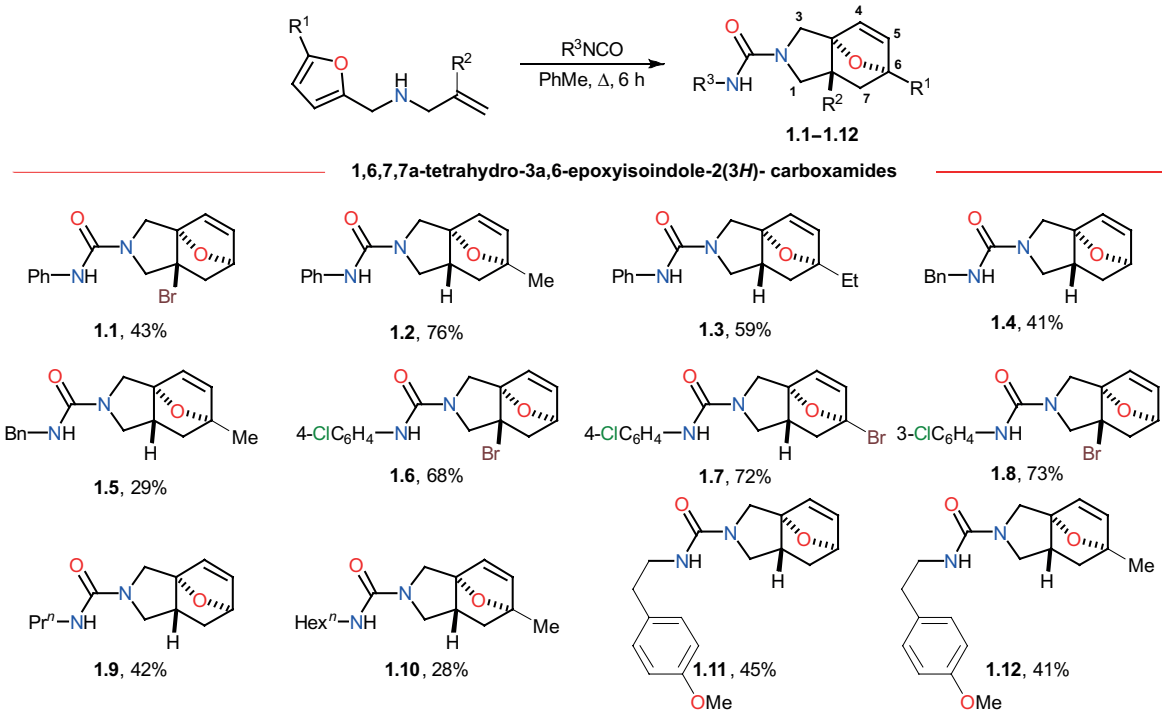

Figure 2 – Scheme of obtaining and structural formulas of hydrogenated 3a,6-epoxyisoindolo-2(3*H*)-carboxamides.

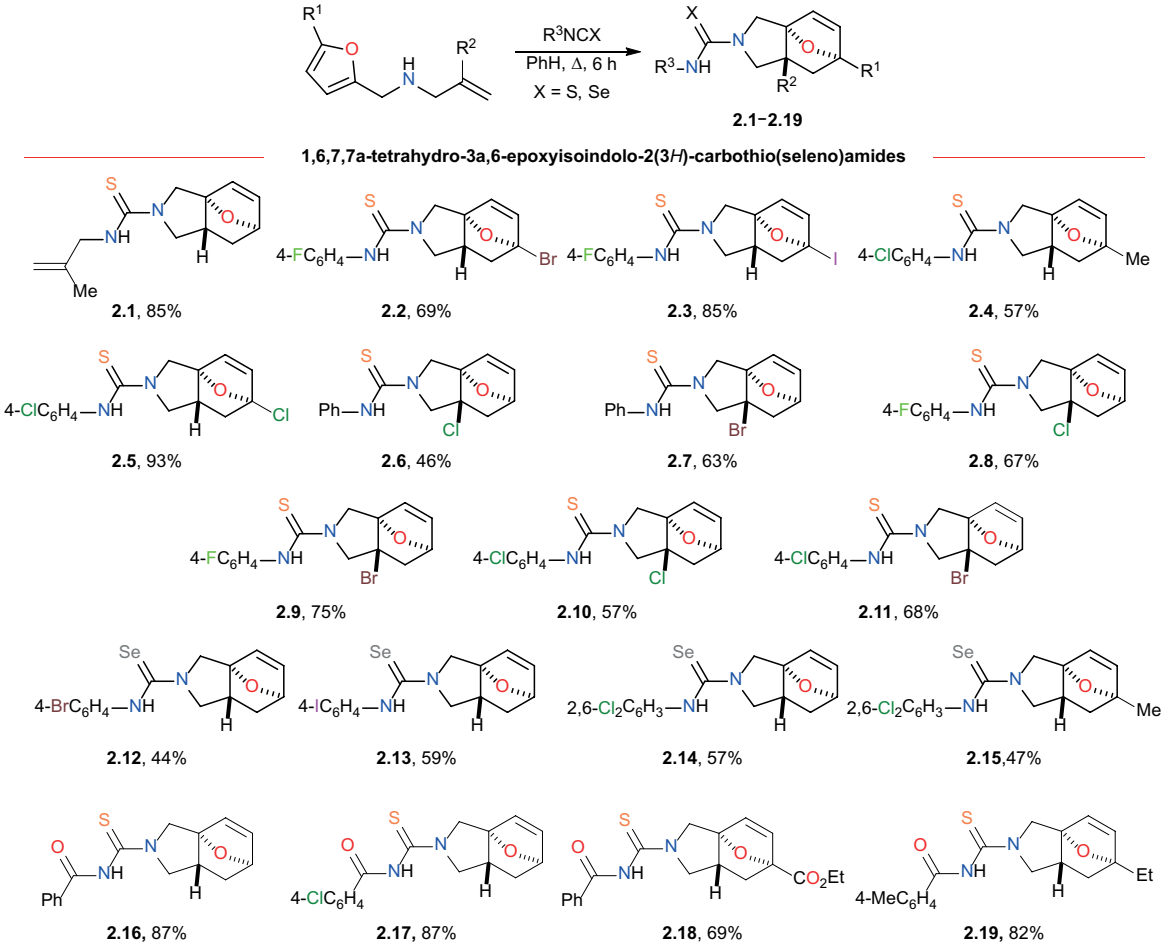

Figure 3 — Scheme of obtaining and structural formulas of hydrogenated 3a,6-epoxyisoindolo-2(3H)-carbothio(seleno)amides.

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2025-13-6-500-514

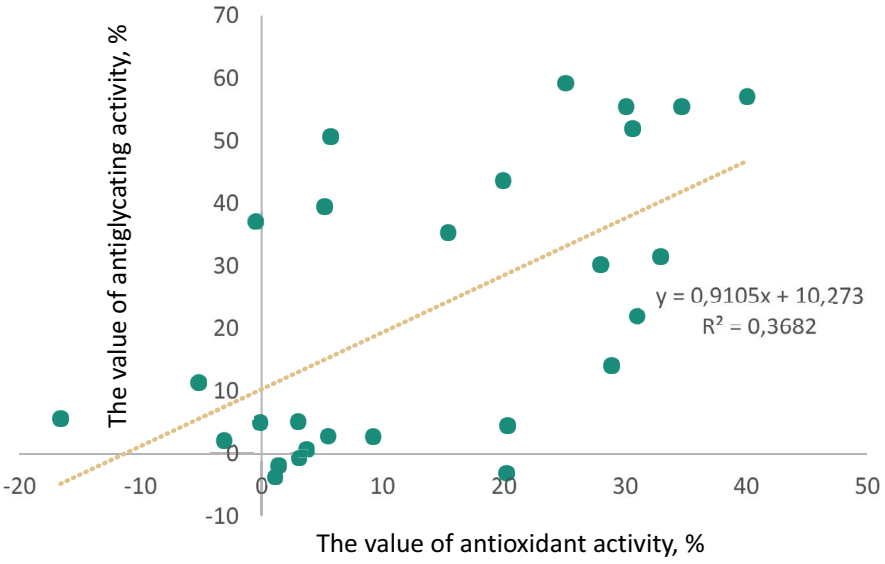

Figure 4 — Graphical representation of the correlation between the values of antiglycating and antioxidant activities of compounds, excluding 2.16, 2.17, 2.18, 2.19 and references.

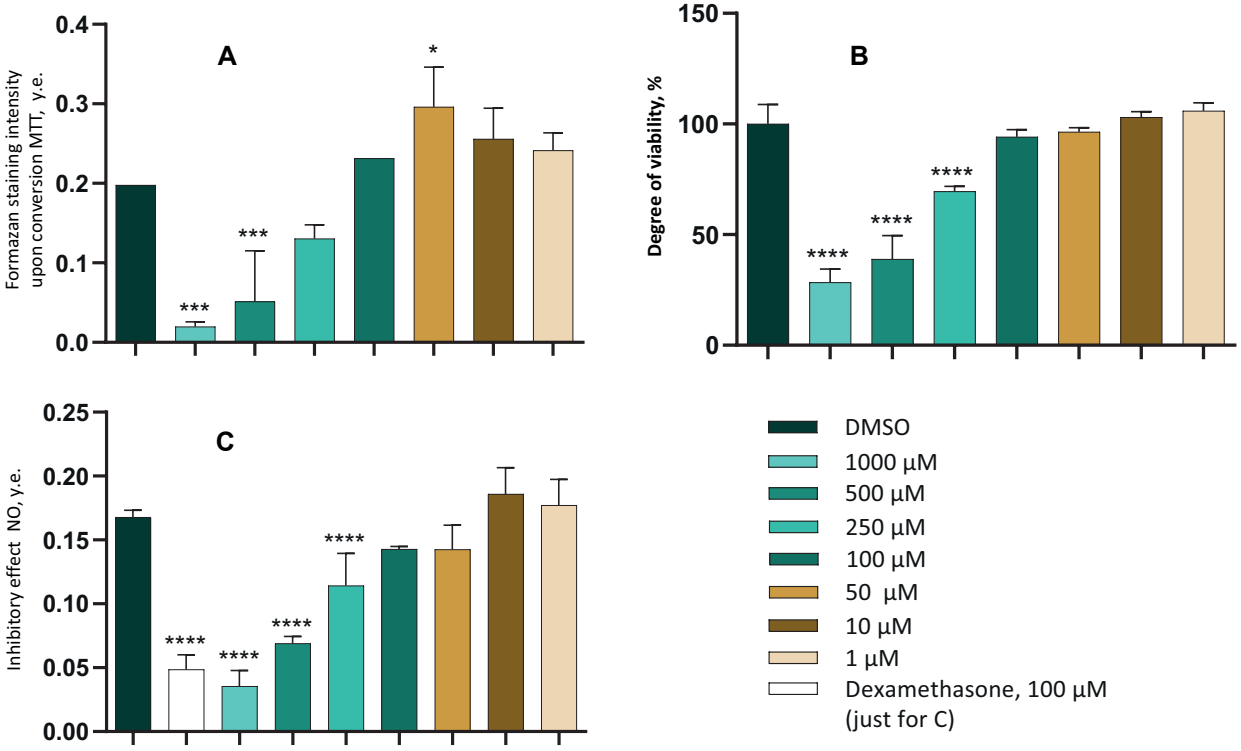

Figure 5 – Results of assessing the cytotoxic and anti-inflammatory properties of compound 2.10.
Note: A — cytotoxic effect of compound 2.10 in the MTT test on peritoneal macrophages; B — cytotoxic effect of compound 2.10 in the test with the determination of released lactate dehydrogenase; C — inhibitory effect of compound 2.10 on NO production caused by the action of lipopolysaccharide compared to dexamethasone. * — statistically significant differences compared to the control group results, ANOVA (Dunnett's post-test: * — $p < 0.05$; ** — $p < 0.01$; *** $p < 0.001$, **** — $p < 0.0001$).

Table 1 – Antiglycating activity of 3a,6-epoxyisoindole derivatives in vitro at a concentration of 100 μM

Compound	Activity, % (M ± SEM)
1.1	-3.1 ± 1.2
1.2	9.2 ± 4.5
1.3	3.7 ± 2.7
1.4	-0.1 ± 3.6
1.5	3.0 ± 2.7
1.6	20.2 ± 2.4*
1.7	5.5 ± 8.5
1.8	1.1 ± 3.1
1.9	-16.6 ± 2.2**
1.10	20.3 ± 2.2**
1.11	1.4 ± 3.5
1.12	3.1 ± 4.3
2.1	-5.2 ± 2.2
2.2	25.1 ± 4.3**
2.3	19.9 ± 3.3*
2.4	-0.5 ± 1.8
2.5	30.1 ± 5.2***
2.6	15.4 ± 1.6****
2.7	5.2 ± 1.6
2.8	34.7 ± 3.1****
2.9	5.7 ± 3.5
2.10	40.1 ± 1.7****
2.11	30.6 ± 3.3***
2.12	32.9 ± 2.3****
2.13	28.0 ± 1.1****
2.14	28.9 ± 2.7****
2.15	31.0 ± 0.9****
2.16	8.8 ± 2.7**
2.17	14.7 ± 1.0**
2.18	1.0 ± 4.1
2.19	-1.0 ± 3.2
Alagebrium	-12.0 ± 1.2
Alagebrium **(1000** μM**)**	15.9 ± 1.5****

Note: * — statistically significant differences compared to the control group results, ANOVA (Dunnett's post-test: * — $p < 0.05$; ** — $p < 0.01$; *** — $p < 0.001$, **** — $p < 0.0001$).

Table 2 – Antioxidant activity of 3a,6-epoxyisoindole derivatives in vitro at a concentration of 111 μM

Compound	Activity, % (M ± SEM)
1.1	2.1 ± 0.6
1.2	2.7 ± 0.7
1.3	0.7 ± 0.4
1.4	5.0 ± 0.2**
1.5	5.1 ± 0.4**
1.6	-3.1 ± 0.7
1.7	2.8 ± 0.7
1.8	-3.7 ± 0.4
1.9	5.6 ± 0.6***
1.10	4.5 ± 0.6
1.11	-1.9 ± 0.5
1.12	-0.7 ± 1.4
2.1	11.4 ± 0.6****
2.2	59.2 ± 0.9****
2.3	43.7 ± 0.7****
2.4	37.1 ± 1.1****
2.5	55.5 ± 0.9****
2.6	35.3 ± 0.7****
2.7	39.5 ± 0.5****
2.8	55.5 ± 0.3****
2.9	50.7 ± 0.8****
2.10	57.1 ± 1.1*****
2.11	52.0 ± 1.3****
2.12	31.5 ± 0.5****
2.13	30.2 ± 1.0****
2.14	14.1 ± 1.2****
2.15	22.0 ± 1.1****
2.16	95.3 ± 0.1****
2.17	96.5 ± 0.2****
2.18	95.0 ± 0.5****
2.19	96.1 ± 0.1****
Quercetin	92.8 ± 1.8****

Note: * — statistically significant differences compared to the control group results, ANOVA (Dunnett's post-test: * — $p < 0.05$; ** — $p < 0.01$; *** — $p < 0.001$, **** — $p < 0.0001$).

Based on the combination of two activities, antiglycation and antioxidant, the best compound was selected from the remaining compounds, which exhibited both desired types of action to the greatest extent simultaneously, namely compound **2.10**, while combining them in a balanced manner. Further studies were conducted with the selected lead.

Evaluation of the cytotoxicity of the lead compound, as well as its anti-inflammatory properties upon induction of inflammation by bacterial lipopolysaccharide

The results of the study of the properties of compound **2.10** in cell models are presented in Figure 5. According to two tests (MTT test and determination of released LDH), the cytotoxic effect of compound **2.10** was detected only at concentrations ≥250 μM (Figures 5A and 5B). This suggests that significant cytotoxicity is absent at lower concentrations of the compound. At the same time, according to the results of the MTT test, a slight cytovitalizing effect is observed at a concentration of 50 μM (Figure 5A).

At the final stage of the study, on a model of LPS-induced NO production, the ability of compound **2.10** to exhibit anti-inflammatory action was evaluated. As a result of this study, it was possible to show the presence of signs of apparent anti-inflammatory activity in the studied compound at

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2025-13-6-500-514

concentrations ≥250 μM (Figure 5C). Such an effect, if reliably confirmed, could be of interest, since suppressing low-level chronic inflammation caused by primary damaging factors is an attractive strategy for preventing fibrosis [36]. Based on the data obtained, compound **2.10**, if it had significantly less activity compared to dexamethasone, but real activity, could be classified as substances with a moderate modulating effect on inflammatory processes. However, comparing the results of the cytotoxicity assessment with the data on the anti-inflammatory activity of **2.10** significantly reduces the likelihood of this type of action in the compound, allowing us to consider the observed effect as false positive, due to cytotoxic properties.

At the same time, this result does not disprove the very connection between primary molecular damage by glycation and oxidative stress with the induction of a secondary cellular response by this exposure, since these triggering factors were absent in the cell model, and instead of glycation or oxidation products, the cells were exposed to bacterial LPS. Thus, further search for compounds combining a complex etiotropic and pathogenetic action, aimed at preventing the development of disabling consequences of difficult-to-treat remodeling fibrotic diseases, as well as their prevention and treatment, should be continued. Compound **2.10** can be considered as a successful intermediate step towards the creation of these compounds.

DISCUSSION

Glycation of proteins and oxidative stress are associated with a whole range of diverse disorders, including pathologies of the cardiovascular system, neurodegenerative diseases [37, 38], as well as conditions associated with tissue fibrosis [10, 39, 40]. Thus, glycation, cross-linking of extracellular matrix proteins, and increasing its rigidity support fibrogenesis [41–44], and excessive production of reactive oxygen species activates profibrotic signaling pathways, including those associated with TGF-β [45]. In addition to this, glycation and oxidative stress provoke inflammatory reactions significant for the course of fibrosis [46, 47]. Based on this, controlling glycation and oxidative stress can effectively complement the range of therapeutic strategies used in the treatment and prevention of fibrosis, thereby increasing the frequency of favorable outcomes.

Antiglycation and antioxidant compounds were searched for among various classes of molecules, both synthetic and natural [48, 49]. The combination of the two indicated activities within one agent is known and has already been considered successful [50, 51]. At the same time, despite the known connection between antiglycation and the manifestation of antioxidant properties [52], these two types of action may not be fully identical, and there are examples (resveratrol and its derivative — triether with trolox) when, upon modification of the compound's structure, a decrease in one activity was accompanied by an increase in the other [53]. At the same time, there are examples (carnosine, hydroxytyrosol) when the combination of these types of action was supplemented with anti-inflammatory properties [54, 55].

The general conclusion from the above is that obtaining new multimodal scaffolds, which in a balanced way combine antiglycation action with antioxidant properties, optionally supplemented with mechanistically related or unrelated anti-inflammatory activity, is a promising strategy in the development of new antifibrotic compounds. In our work, we focused on the construction of bidirectionally active compounds that combine the ability to antiglycation with antioxidant activity — based on an epoxyisoindole scaffold, namely in the series of 3a,6-epoxyisoindole-2(3*H*)-(carbox/thio/seleno)amides. This molecular basis can be considered privileged due to the fact that it is (i) available through stereoselective IMDAF sequences, allowing for a wide variation of introduced (chalcogen)carbamoyl fragments, and (ii) has already demonstrated biological prospects in related tasks. Thus, it has been shown that the IMDAF approach allows obtaining a 3a,6-epoxyisoindole core in a small number of synthetic steps, which facilitates the generation of a voluminous library of compounds for subsequent analysis of the dependence of activity on structure. Structurally similar isoindole derivatives have previously demonstrated antiglycation properties [56], which indirectly supports the hypothesis about the prospects of the epoxyisoindole core as a matrix for constructing compounds that combine antiglycation and antioxidant activity. Additional privilege to the epoxyisoindole base is added by its wide distribution in natural and drug-like compounds with various biological effects [57].

The study showed that the most balanced combination of antiglycation and antioxidant activities in the series of studied compounds is possessed by

(3aRS,6SR,7aRS)-7a-chloro-*N*-(4-chlorophenyl)-1,6,7,7a-tetrahydro-3a,6-epoxyisoindole-2(3*H*)-carbothioamide (compound **2.10**). Combining both activities, expressed at a sufficient level for their further optimization, the compound does not demonstrate the prevalence of one of them. The molecule contains fragments of thiourea, hydrogenated isoindole, and halogenarene in its structure. It is interesting that separately or in other combinations, these fragments are present in other compounds of the series, for example, thiourea in **2.1–2.9** and **2.11**, and a para-chlorophenyl substituent in structures **2.11** and **2.17**. But it is the combination of all available structural fragments that makes **2.10** an outstanding representative of the series. This is important, since the desired scaffold must bear signs of integrity, and its further modifications may be aimed primarily at including substituents in points of its structure that are insignificant for the final pharmacological activity.

A fundamental feature of compound **2.10** and the entire studied series is the absence of phenolic hydroxyl groups in their structures, traditionally associated with antioxidant and antiglycation activity. Apparently, this limits the potential magnitude of the observed effects in the models used. But this is also justified by the fact that the compounds under consideration are positioned primarily as starting frameworks for subsequent structure optimization — at the current stage, the goal of the work was to find a new scaffold of a higher level (more complexly organized than the original epoxyisoindole), suitable for further enhancing its antiglycation and antioxidant properties, and not in achieving maximum activity values.

At the same time, one cannot speak about the balance of the combination of the two desired types of action when discussing the established properties of **2.16**, **2.17**, **2.18**, and **2.19**. Unlike all other representatives of the studied series, these compounds contain an N-aroyl fragment in their structure, which, obviously, contributes to the manifestation of pronounced antioxidant properties. At the same time, due to the discrepancy between antioxidant and antiglycation actions, the phenomenon of high antioxidant activity of these compounds has a low final significance. At the same time, the properties of the four indicated compounds may be of interest for future research.

The final stage of the discussion concerns the anti-inflammatory activity and overall safety of the lead compound **2.10**. In the LPS-induced inflammation model used on PMs, specific anti-inflammatory activity of **2.10** was not confirmed; the reduction in NO production observed in the study was primarily seen at concentrations where cytotoxic effects were observed (≥250 μM), while no significant effect was found in the subtoxic concentration range. At the very least, the observed result indicates that compound **2.10** is not characterized by the inhibition of intracellular inflammatory cascades. The latter could be valuable for the suppression of fibrogenesis. At the same time, we note that due to the absence in the model of a component associated with the induction of an inflammatory reaction by certain damaged molecular patterns (e.g., glycated protein) capable of acting as triggers for NO production (LPS was used instead), further clarifying studies are needed. Regarding safety, the combined result of the two cytotoxicity assessment methods indicates a likely acceptable tolerability of **2.10**, although the translation of the results of cellular toxicity studies is complex and will require more complexly organized biological models in the future.

Thus, this work defines the epoxyisoindole scaffold as a promising basis for creating compounds with a combination of antiglycating and antioxidant properties, presents promising directions for modifying the original epoxyisoindole scaffold for the development of new drugs, and identifies the found higher-order Scaffold — compound **2.10** — as a balanced starting candidate for further structure optimization. In methodological terms, the work describes a strategy for finding means for the prevention and treatment of pathologies dependent on the trigger mechanisms of damage by glycation and oxidative stress, including fibrotic diseases.

Study limitations

Despite all the advantages, this study has several limitations:

1. The study is exploratory and pilot in nature.
2. Despite the sufficient justification for the relationship between changes in the extracellular matrix under the influence of glycation, as well as oxidative stress and inflammation, with the mechanisms of fibrogenesis, the work lacks direct endpoints reflecting the dynamics of fibrotic changes, including models of tissue/organ fibrosis. Consequently, conclusions about

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2025-13-6-500-514

antifibrotic potential are logically justified but inferential in nature.

3. All experimental models used in the study are in vitro and in cellulo. When assessing antiglycating properties, the study is limited to one model protein (BSA), and cellular studies are limited to one type of cell (primary mouse PMs).
4. Despite the measures taken to reduce methodological interference in test systems, it is impossible to completely exclude it. The anti-inflammatory effect of the lead compound has not been confirmed, and the observed effect is associated with cytotoxicity.
5. The stability of the studied compounds under the conditions of the test systems used was not checked and was not the subject of the study.

CONCLUSION

According to the results of the study, compound **2.10** ((3aRS,6SR,7aRS)-7a-chloro-*N*-(4-chlorophenyl)-1,6,7,7a-tetrahydro-3a,6-epoxyisoindole-2(3*H*)-carbothioamide) combines antiglycating and antioxidant activity in the absence of significant cytotoxicity. However, no anti-inflammatory activity was found in compound **2.10**. Despite this, the established activity profile indicates the potential of the compound as a higher-order molecular basis (than the original epoxyisoindole), suitable for the directed design of new means for the prevention and treatment of conditions associated with the damaging effects of glycation and oxidative stress, especially means for the prevention and treatment of fibrotic diseases.

FIUNDING

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS CONTRIBUTION

Umida M. Ibragimova — methodology, data analysis, investigation, project administration, writing—original draft, writing—review & editing, visualization; Nikita V. Valuysky — methodology, data analysis, investigation, project administration, writing—original draft, writing—review & editing; Violetta R. Rayberg, Svetlana A. Sorokina, Ksenia I. Zhukova, Denis K. Deryagin, Ilya S. Ukhorenko, Alesya A. Grigoryeva, Dmitry M. Shchevnikov — methodology, data analysis, investigation; Vladimir P. Zaitsev — methodology, data analysis, investigation, writing—original draft, writing—review & editing; Roman A. Litvinov — conceptualization, methodology, resources, project administration, writing—original draft, writing—review & editing, supervision, funding acquisition.

All authors confirm that their authorship meets the international ICMJE criteria (all authors have made significant contributions to the development of the concept, research and preparation of the article, read and approved the final version before publication).

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